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Engineering High Catalytic Efficiency of the Steroid Isomerase Activity of Human Glutathione *S*-transferase P1-1

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Glutathione *S*-transferases (GSTs) are a family of multifunctional enzymes that catalyze the formation of conjugates between reduced glutathione (GSH) and a variety of carcinogenic, mutagenic, toxic and pharmacologically active compounds.[1,2] GSTs have also been shown to participate in other biological process. Certain GSTs can detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity, while others can catalyze the isomerization of certain steroids. Mammalian cytosolic GSTs, which exist as homo- or heterodimers, are grouped into at least seven distinct classes - alpha, mu, pi, sigma, theta, sigma, kappa and zeta - according to their physical, chemical, immunological and structural properties.[1,3,4]

Human pi-class hGST P1-1 contains eight arginine residues. Among them, Arg13 is the only residue conserved in all the known Pi class GSTs and in most of the Alpha class GSTs. The highly conserved residues are expected to be important to the enzyme's structure and function. hGST P1-1 is particularly interesting as it can be used as a reliable preneoplastic or neoplastic marker because it is expressed at raised levels by a number of human tumors.[5,6] It has also been implicated in the development of tumors' resistance to various anti-cancer drugs.[7,8] Study of the enzyme's structure-function relationships could aid the design of inhibitors and prodrugs with enhanced therapeutic indices. Therefore, the catalytic mechanism of hGST P1-1 has been studied through chemical modification and site-directed mutagenesis. Three-dimensional structures of hGST P1-1 have used to implicate several amino acid residues in catalysis and the binding of substrates,[9-13] elucidating the precise enzyme-substrate interactions responsible for catalysis. Arginine residues have been suggested to be located at or near GSTs' active sites and are likely essential to catalysis.[10-12] The contribution of Arg13 in the substrate binding of hGST P1-1 is highly dependent on the nature of the electrophilic substrate and this residue participates in the catalytic mechanism mainly through the construction of the correct conformation of the active site; it also contributes to the binding of the electrophilic substrates.[14,15]

This work reports the increased catalytic efficiency of the steroid isomerase activity by the redesign of the enzyme-substrate interactions responsible for the GSH peroxidase and steroid isomerase activities of GST. Mutant enzymes obtained by the site-directed mutagenesis of Arg13 in hGST P1-1 into Ala, Lys, and Leu were expressed in *E. coli* and purified to electrophoretic homogeneity by affinity column chromatography. The kinetic parameters and thermal stabilities of the mutants and the wild-type were compared. Two different substrates, cumene hydroperoxide and androstenedione, were chosen to study the effects of the mutations on two chemical reactions that the enzyme can catalyze: GSH peroxidase activity and steroid isomerase activity.

The GST-catalyzed reaction with cumene hydroperoxide represents "nonselenium" glutathione peroxidase activity and is believed to occur in two steps involving an unstable glutathione sulfenic acid intermediate (Figure 1(a)). Reduced GSH is then regenerated from the produced glutathione disulfide (GSSG) by the action of glutathione reductase.[1] The kinetic parameters of the mutant enzymes for GSH peroxidase activity are shown in Table 1. Substitutions of Arg13 with alanine (R13A) and leucine (R13L) significantly

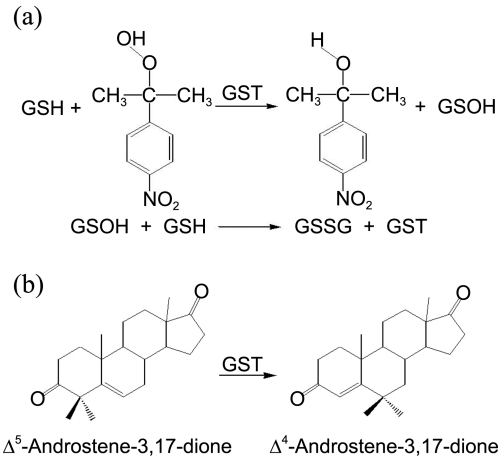

Figure 1. GSH peroxidase and steroid isomease activities in GST-catalyzed reactions. (a) GSH peroxidase activity towards cumene hydroperoxidase; (b) Steroid isomerase activity towards Δ^5-Androstene-3,17-dione.

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CP, cumene hydroperoxide; GSH, glutathione; G-site, glutathione-binding site; GST, glutathione *S*-transferase; hGST, human GST; H-site, electrophilic substrate-binding site.

Table 1. Specific activities and kinetic parameters for GSH peroxidase activity towards cumene hydroperoxide

Enzyme	GSH			Cumene hydroperoxide		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type	0.17 ± 0.03	1.37 ± 0.07	8.06	0.30 ± 0.02	0.96 ± 0.04	3.20
R13A	3.60 ± 0.02	0.96 ± 0.11	0.27	1.30 ± 0.01	0.77 ± 0.11	0.59
R13K	0.12 ± 0.06	1.50 ± 0.06	12.50	0.25 ± 0.04	1.28 ± 0.03	5.12
R13L	0.26 ± 0.09	1.38 ± 0.03	5.31	1.57 ± 0.10	0.30 ± 0.92	0.19

Values are Means ± S.D., generally based on n ≥ 5.

Table 2. Specific activities and kinetic parameters for steroid isomerase activity towards Δ^5-androstene- 3,17-dione

Enzyme	GSH			Δ^5-androstene- 3,17-dione		
	K_m (mM)	k_{cat} ($\times10^{-2}$ s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} ($\times10^{-2}$ s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type	0.88 ± 0.04	2.4 ± 0.3	0.027	0.43 ± 0.09	15.4 ± 3.2	0.36
R13A	1.00 ± 0.06	3.4 ± 0.5	0.034	0.44 ± 0.01	5.2 ± 1.0	0.12
R13K	0.18 ± 0.01	6.6 ± 0.8	0.367	0.22 ± 0.06	130.5 ± 9.6	593.18
R13L	1.30 ± 0.05	0.2 ± 0.01	0.002	1.46 ± 0.12	0.4 ± 0.02	0.003

Values are Means ± S.D., generally based on n ≥ 5.

altered the substrate binding sites. The substitution with alanine increased K_m^{GSH} approximately 21 times, whereas K_m^{CP} and k_{cat} remained similar to those of the wild type. The substitution with leucine also resulted in a five-fold increase of K_m^{CP}. The substitution of Arg13 with lysine increased k_{cat} approximately 1.3 times for cumene hydroperoxide, giving a catalytic efficiency (k_{cat}/K_m^{CP}) 1.6 times that of hGST P1-1. These results suggest that the negative charge in position 13 participates in GSH peroxidase catalysis and that the size of the side chain significantly affects substrate binding.

GST isozymes can also possess ketosteroid isomerase activity and catalyze the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids in the biosynthesis of progesterone and testosterone (Figure 1(b)). The kinetic parameters for the steroid isomerase activity of R13A or R13L mutants are shown in Table 2. Both R13A and R13L decreased k_{cat} approximately three times and 39 times, respectively. R13L mutant approximately trebled $K_m^{STEROID}$, and decreased $k_{cat}/K_m^{STEROID}$ by a factor of approximately 130 (Figure 3). R13K mutant significantly increased the catalytic efficiency for steroid isomerase activity: $K_m^{STEROID}$ halved; k_{cat} increased approximately 8.5 times and $k_{cat}/K_m^{STEROID}$ increased approximately 17 times. The incresed catalytic efficiency was due to the smaller side chain at position 13 in hGST P1-1. Substitution to lysine is thought to increase the affinity for Δ^5-androstene 3,17-dione, possibly by providing sufficient space for the binding of Δ^5-androstene 3,17-dione and/or by changing the conformation of the active site and also by permitting the proper orientation of the bound substrates.

The mutants' stabilities were assessed by measuring the temperature at the midpoints of heat inactivation (Figure 4). The wild type and R13L mutants showed midpoints of inactivation at approximately 56 °C. The temperature of the R13K mutant was decresed to approximately 51 °C. The

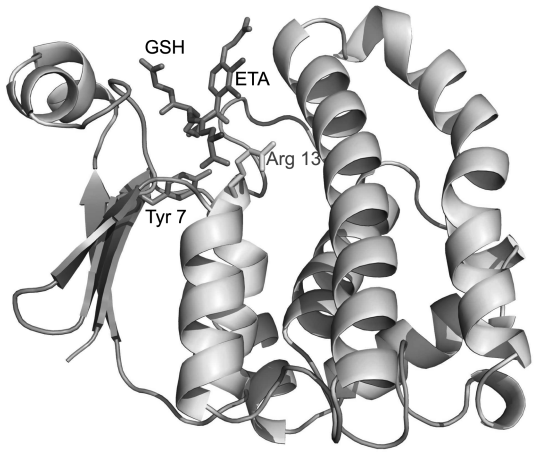

Figure 2. Magnified view of the active site of human GST P1-1.[13] Ball-and-stick representation of the active site of human GST P1-1 showing the location of the GSH-ethacrynic acid (ETA) complex bound to Tyr7 and Arg13. The figure was generated with MOLSCRIPT.

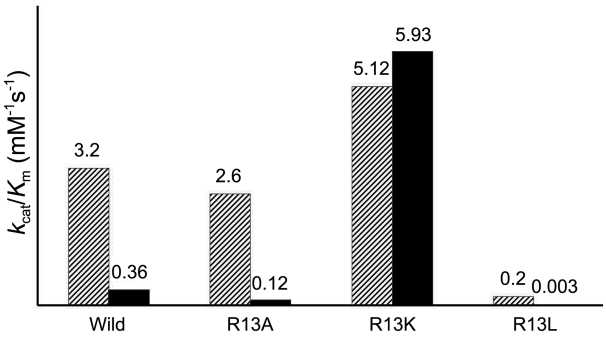

Figure 3. Substrate selectivity profiles expressed as catalytic efficiencies, k_{cat}/K_m, of the wild type and mutant enzymes with the following substrates: cumene hydroperoxide (hatched) and Δ^5-androstene-3,17-dione (black). The kinetic parameters are from Tables 1 and 2.

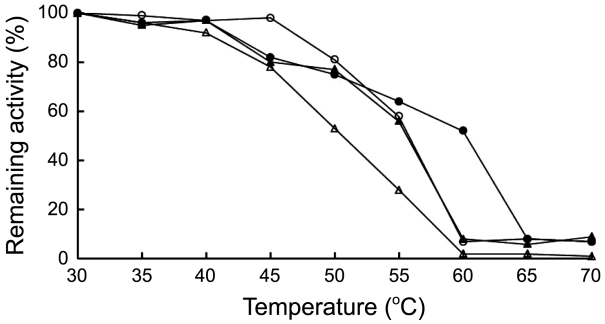

Figure 4. Thermostabilities of the wild type and mutant enzymes. Wild type, ○; R13A, ●; R13K, △; R13L, ▲.

R13A mutant was inactivated at the increased temperature of approximately 60 °C, indicating the most stable enzyme among them. These results suggest that the substitution of Arg13 with lysine resulted in a little conformational change of the active site of hGST P1-1.

GSTs have been shown to participate in the biosynthesis of steroid hormones. The ketosteroid isomerase activity of GST was discovered in a rat liver cytosol fraction. A mechanism for the steroid isomerase activity of GST, in which the thiolate of glutathione serves as a base, has been proposed for GST A3-3 on the basis of the kinetics and pH dependence of the double-bond isomerization of Δ^5-androstene-3,17-dione.[16] It was supported by the crystal structure of GST A3-3 and by the model of the ternary complex enzyme/glutathione/Δ^5-androstenedione.[17] Alpha-class human GST A3-3 is the most efficient steroid isomerase known in human tissues for the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids. It has been shown to possess exceptionally high steroid double-bond isomerase activity with Δ^5-androstene-3,17-dione (specific activity: 197 μmol/mg/min). The specific activity of hGST P1-1 towards Δ^5-androstene-3,17-dione has been found to be lower, approximately 78 μmol/mg/min.[18]

The evolution of proteins for novel functions involves point mutations of the active site residues. Mimicking this process *in vitro* to engineer proteins by rational design remains a major challenge. This work showed that the substitution of Arg13 with lysine significantly reduced steroid isomerase activity towards Δ^5-androstene 3,17-dione, increasing $k_{cat}/K_m^{STEROID}$ by two orders of magnitude (Table 2 and Figure 3). The R13K mutant of hGST P1-1 also showed approximately 4-fold increase of specific activity (368 μmol/mg/min) towards Δ^5-androstene 3,17-dione. The R13K mutant and the wild-type enzyme showed similar activities towards CDNB[15] and cumene hydroperoxide (Table 1). These changes of catalytic properties were most likely correlated with structural differences between the active sites, and substitution of Arg13 to lysine likely affected interactions between the thiol group and Tyr7 through distorting the orientation of the GSH bound in the enzyme and/or distorting the binding site of Δ^5-androstene 3,17-dione. The active-site residue Arg13 is conserved in all known pi class GSTs and in most alpha class GSTs. Arg13 in hGST P1-1, equivalent to Arg15 in class alpha GSTs, was positioned close to the xenobiotic substrate and appeared to be crucial to the high steroid isomerase activity. This is supported by three dimensional crystal structures of hGST P1-1. Reinemer *et al*. suggested that the electrophilic substrate-binding site (H-site) of hGST P1-1 is located adjacent to the glutathione-binding site (G-site), with the hexyl moiety of *S*-hexyl-GSH bound in the enzyme lying above a segment connecting strand β1 and α-helix A.[10] The three-dimensional structure of human GST P1-1 in complex with ethacrynic acid, an inhibitor, suggested that the inhibitor is located in a hydrophobic pocket lined with the side chains of Tyr7, Phe8, Pro9 and Val10 and the aliphatic portions of Arg13, Val35, Ile104 and Tyr108; and that the carboxylic acid moiety of the inhibitor forms a hydrogen bond with the N_ε atom of Arg13 (Figure 2).[13] The H-site must be adjacent to the G-site, and should also permit proper orientation of the bound reactants. Therefore, substitution of Arg13 with lysine appeared to change the conformation of the active site, increasing affinity for GSH and Δ^5-androstene 3,17-dione. Lysine residue having a positive charge at position 13 may also be able to participate in steroid isomerase catalysis. Introduction of a residue with a smaller side chain in this position improved affinity (Lys > Arg) towards the larger substrate like a Δ^5-androstene 3,17-dione by allowing the construction of a proper electrostatic field; and achieved an active site conformation with high catalytic efficiency for steroid isomerase activity.

In conclusion, the evolutionally-conserved Arg13 residue in hGST P1-1 was demonstrated to be an important residue that modulates the enzymes' catalytic efficiency and specificity; mutation of this residue to Lys was shown to allow tailoring the conformation of the active site of the enzyme for high catalytic efficiency with Δ^5-androstene-3,17-dione.

Experimental Section

Materials. GSH and 1-chloro-2,4-dinitrobenzene were from Kohjin Co. and Wako Pure Chem. Ind. (Osaka, Japan), respectively. Cumene hydroperoxide was from Sigma (St. Louis, USA). Δ^5-androstene-3,17-dione was from Steraloids Inc. (Wilton, N. H. USA). Glutathione Sepharose was from Pharmacia Biotech (Uppsala, Sweden). All other reagents were of the highest grade commercially available.

Preparation of Mutant Enzymes. Wild-type hGST P1-1 was obtained from the expression of cDNA in *E. coli* as described elsewhere.[19] The mutants R13A, R13K and R13L were prepared, confirmed, expressed and purified by established methods,[14] with the exception of purification by affinity chromatography on GSH-Sepharose. The mutant enzymes were expressed in *E. coli* under the control of the *tac* promoter. Cultured cells were lysed and centrifuged. The dialyzed supernatant of the cell lysate was loaded directly onto a 15 mL column of GSH-Sepharose equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). The column was extensively washed with buffer A containing 20

mM KCl. The enzyme was eluted with 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM GSH and dialyzed against buffer A. The dialyzed purified enzyme was in subsequent tests. Unless otherwise indicated, all purification procedures were carried out either at 4 °C or on ice. The enzymes were stored at −70 °C until used.

Enzyme Activity. GSH-dependent peroxidase activity was assayed in the presence of 5 mM GSH with 1.5 mM cumene hydroperoxide ($\Delta\varepsilon$ = 6.6 $mM^{-1}cm^{-1}$) substrate at 30 °C, as described elsewhere.[20] Steroid isomerase activity was monitored by the catalyzed isomerization of Δ^5-androstene-3,17-dione (0.1 mM) to Δ^4-androstene-3,17-dione, and was determined spectrophotometrically from the change in absorbance at 248 nm ($\Delta\varepsilon$ = 16.3 $mM^{-1}cm^{-1}$) in 100 mM potassium phosphate buffer, at pH 6.5 and at 30 °C in the presence of 3 mM GSH. Protein concentration of the wild type enzyme was determined by measuring absorbance at 280 nm, as described by Parker,[21] and protein concentration of the mutant was determined using protein assay reagent (Bio-Rad Lab.) and the wild-type enzyme as a standard protein.

Kinetic Studies. Kinetic studies with GSH and electrophilic substrates were carried out at 30 °C as described elsewhere.[22] Kinetic parameters, K_m, were determined under first-order conditions at low substrate concentrations: for GSH with a fixed concentration of 1.5 mM cumene hydroperoxide or Δ^5-androstene-3,17-dione; for cumene hydroperoxide or Δ^5-androstene-3,17-dione with a fixed concentration of 5 mM GSH. k_{cat} was calculated on the basis of mol dimeric enzyme using a Mr of 45,000. Other experimental conditions were similar to those used in the determination of specific activities.

Heat Inactivation Assays. Enzymes were incubated at each temperature for 10 min at a protein concentration of 0.1 mg/mL in 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM DTT and 10 mM EDTA to prevent oxidative inactivation, and then cooled in ice. The remaining activity was assayed in 100 mM potassium phosphate buffer (pH 6.5) with 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene at 30 °C. The enzyme was incubated in buffer A at various temperatures for 10 min and then cooled on ice.

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